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MECHANISMS OF RESISTANCE

Low Prevalence of Gram-Positive Isolates Showing Elevated Lefamulin MIC Results during the SENTRY Surveillance Program for 2015–2016 and Characterization of Resistance Mechanisms

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ABSTRACT This study investigated the molecular mechanisms possibly associated with non-wild-type MICs for lefamulin among staphylococci and streptococci included in the lefamulin surveillance program from 2015 to 2016. A total of 2,919 *Staphylococcus aureus*, 276 coagulase-negative staphylococci (CoNS), 3,923 *Streptococcus pneumoniae*, 389 β-hemolytic, and 178 viridans group streptococci isolates were included in the surveillance studies. Eleven (0.3% of all *S. aureus*) *S. aureus* isolates with lefamulin MICs above the staphylococcal epidemiological cutoff (ECOFF) value (>0.25 μg/ml) were selected for this study. Eight (72.7%) *S. aureus* (lefamulin MIC, 0.5 to 4 μg/ml) isolates carried *vga*(A or E), one isolate (MIC, 32 μg/ml) carried *lsa*(E), one isolate (MIC, 16 μg/ml) had an alteration in L4, and one strain (MIC, 0.5 μg/ml) did not carry any of the investigated resistance mechanisms. A total of 14 (5.1% of all CoNS) CoNS isolates had lefamulin MICs (0.5 to >32 μg/ml) above the ECOFF. Similar to *S. aureus*, 8 (57.1%) CoNS (lefamulin MIC, 1 to 8 μg/ml) isolates carried *vga*(A or B), while 2 isolates (MIC, 4 to 32 μg/ml) carried *cfr*. High genetic diversity was observed among staphylococci, although 3 *S. aureus* isolates belonged to sequence type 398 (ST398). Among the 3 *Streptococcus agalactiae* and 3 viridans group streptococci (0.1% of all streptococci surveyed) isolates selected for additional characterization, all but 1 isolate carried *lsa*(E). This study documents a low occurrence of surveillance isolates exhibiting a non-wild-type MIC for lefamulin, and among these isolates, *vga* and *lsa*(E) prevailed in staphylococci and streptococci, respectively.

KEYWORDS BC-3781, pleuromutilins, *vga*(A), *lsa*(E)

Lefamulin belongs to the pleuromutilin class of antibiotics, and its antibacterial profile covers the most relevant organisms causing community-acquired bacterial pneumonia (CABP), including Gram-positive, fastidious Gram-negative, and atypical respiratory pathogens (1–3). Lefamulin also shows *in vitro* activity against multidrug-resistant *Neisseria gonorrhoeae* and *Mycoplasma genitalium* (4, 5). Thus, in addition to the clinical utility for treating CABP, the characteristic lefamulin antibacterial profile fits treatment for acute bacterial skin and skin structure infections (ABSSSIs) and sexually transmitted diseases (6).

Lefamulin inhibits bacterial protein synthesis by binding the 23S ribosomal subunit at the A and P sites in the peptidyl transferase center (PTC) via 4 hydrogen bonds and other interactions. An "induced-fit" mechanism, which is characteristic for pleuromutilin antibiotics and causes the tight fit of these molecules to the target site, hinders the correct positioning of the tRNA and thereby prohibits peptide bond formation (7, 8).

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Mechanisms mediating resistance to pleuromutilins include mutations within the domain V of the 23S rRNA, including methylation of the nucleotide A2503 by the methyl transferase Cfr (9). Mutations in the 23S rRNA at positions 2032, 2055, 2447, 2499, 2504, and 2572 were previously described to confer resistance to tiamulin in *Brachyspira* spp. (10), while alterations at positions 2055, 2447, 2504, and 2572 were associated with valnemulin resistance in *Mycobacterium smegmatis* (11). Ribosomal proteins L3 and L4 are not primarily pleuromutilin targets, but mutations within these molecules may alter the PTC structure and affect binding. L3 (*rplC*) at the amino acid positions 145, 148, 149, 152, 155, 157, 158, and 159 and L4 (*rplD*) at position 68 were associated with resistance (7, 10, 12–14). Moreover, ATP-binding cassette F (ABC-F) proteins, such as *vga*(A–E) and *lsa*(E), initially described as putative efflux pumps can cause pleuromutilin resistance by ribosomal protection (15, 16).

As part of the clinical development, the *in vitro* activity of lefamulin and comparator agents have been monitored against a global collection of Gram-positive and fastidious Gram-negative organisms causing CABP and ABSSSI through the SENTRY Antimicrobial Surveillance Program. This study evaluated the occurrence of staphylococci and streptococci displaying elevated lefamulin MICs or above the epidemiological cutoff (ECOFF) during the SENTRY Program from 2015 to 2016 and characterized the possible associated resistance mechanisms among non-wild-type surveillance isolates.

RESULTS

Lefamulin had MIC_{50} and MIC_{90} results of 0.06 and 0.12 μg/ml, respectively, with the majority (99.6%) of isolates displaying MICs of $\leq$0.008 to 0.25 μg/ml (Table 1). Eleven *Staphylococcus aureus* isolates showed lefamulin MICs above the ECOFF value (i.e., $>$0.25 μg/ml), and these isolates represented 0.3% of all *S. aureus* included in the 2015 and 2016 lefamulin surveillance programs (Table 1). When lefamulin was tested against coagulase-negative staphylococci (CoNS), a total of 14 isolates (4 species) had lefamulin MICs (0.5 to $>$32 μg/ml) above the ECOFF value (Tables 1, 2). The lefamulin MIC_{50} results obtained against streptococci varied depending on species or group of species (Table 1), and 3 *Streptococcus agalactiae*, 2 *Streptococcus lutetiensis*, and 1 *Streptococcus gallolyticus* isolates showed lefamulin MICs outside the wild-type distribution for the respective species and were further investigated.

Most *S. aureus* (7/11; 63.6%) isolates displaying lefamulin MICs of $>$0.25 μg/ml harbored *vga*(A) (lefamulin MIC, 0.5 to 4 μg/ml), while 2 strains carried either *vga*(E) (lefamulin MIC, $>$32 μg/ml) or the *lsa*(E) gene (lefamulin MIC, 32 μg/ml) (Table 3). Very little variability was observed in the *S. aureus* 23S rRNA nucleotide and ribosomal sequences. Overall, each isolate contained the same polymorphisms in the 23S rRNA (A21G, A1557T, and/or A2234G), while ribosomal proteins had wild-type sequences. The only exception was observed for isolate 916083, which had a V118A and an E147K in L4 (lefamulin MIC, 16 μg/ml). One *S. aureus* (975498) isolate with a lefamulin MIC of 0.5 μg/ml did not show any known resistance mechanisms associated with pleuromutilins. High genetic diversity was observed among staphylococci, although 3 *S. aureus* strains belonged to sequence type 398 (ST398).

Both CoNS with a lefamulin MIC of 0.5 μg/ml, 1 *Staphylococcus cohnii* and 1 *Staphylococcus epidermidis* isolate, did not contain any known pleuromutilin resistance genes; however, both isolates had multiple alterations in the 23S rRNA or ribosomal proteins. Eight (57.1%) CoNS isolates contained acquired *vga* variants (lefamulin MIC, 1 to 8 μg/ml) (Table 3). The *cfr* gene was detected in 1 *Staphylococcus haemolyticus* (lefamulin MIC, 4 μg/ml) isolate and 1 *S. epidermidis* (lefamulin MIC, 32 μg/ml) isolate. The latter also showed multiple mutations in 23S rRNA, L3, and L4 (Table 3). Two *Staphylococcus sciuri* (lefamulin MIC, 16 to 32 μg/ml) isolates carried the intrinsic putative *sal*(A) gene (Table 3). In general, *S. epidermidis* isolates showed alterations in the 23S rRNA, such as G241T, T669C, and T1236C, that could be considered polymorphisms.

Among all streptococci surveyed, including 3,923 *S. pneumoniae*, 3 *S. agalactiae*, 2 *S. lutetiensis*, and 1 *S. gallolyticus* isolates, those with elevated lefamulin MICs (0.5 to 32

TABLE 1 Lefamulin MICs obtained during surveillance programs for 2015 and 2016[a]

Organism	No. (cumulative %) of isolates with a lefamulin MIC (μg/ml) of:														Total no. of isolates	MIC_{50} (μg/ml)	MIC_{90} (μg/ml)
	≤0.008	0.015	0.03	0.06	0.12	0.25	0.5	1	2	4	8	16	32	>32			
Staphylococcus spp.																	
S. aureus	5 (0.2)	10 (0.5)	745 (26.0)	1,836 (88.9)	300 (99.2)	12 (99.6)	2 (99.7)	3 (99.8)	1 (99.8)	0 (99.8)	0 (99.8)	1 (99.9)	0 (99.9)	4 (100.0)	2,919	0.06	0.12
Coagulase-negative staphylococci	2 (0.7)	42 (15.9)	139 (66.3)	68 (90.9)	8 (93.8)	3 (94.9)	3 (96.0)	2 (96.7)	3 (97.8)	3 (98.9)	2 (99.6)	0 (99.6)	0 (99.6)	1 (100.0)	276	0.03	0.06
Streptococcus spp.																	
S. pneumoniae	5 (0.1)	64 (1.8)	377 (11.4)	1,715 (55.1)	1,513 (93.7)	234 (99.6)	10 (99.9)	5 (100.0)							3,923	0.06	0.12
β-hemolytic streptococci																	
S. agalactiae	1 (0.6)	21 (13.1)	136 (94.0)	4 (96.4)	3 (98.2)	0 (98.2)	0 (98.2)	0 (98.2)	0 (98.2)	0 (98.2)	2 (99.4)	1 (100.0)			168	0.03	0.03
S. pyogenes	5 (3.0)	80 (51.5)	80 (100.0)												165	0.015	0.03
S. dysgalactiae		3 (5.4)	45 (85.7)	8 (100.0)											56	0.03	0.06
Viridans group streptococci																	
S. mitis group		5 (10.4)	3 (16.7)	10 (37.5)	12 (62.5)	11 (85.4)	7 (100.0)								48	0.12	0.5
S. anginosus group	2 (4.5)	7 (20.5)	8 (38.6)	11 (63.6)	10 (86.4)	2 (90.9)	4 (100.0)								44	0.06	0.25
S. salivarius/*S. vestibularis* group	5 (12.5)	4 (22.5)	6 (37.5)	19 (85.0)	5 (97.5)	1 (100.0)									40	0.06	0.12
S. gallolyticus				2 (5.9)	2 (11.8)	3 (20.6)	3 (29.4)	9 (55.9)	13 (94.1)	1 (97.1)	0 (97.1)	0 (97.1)	0 (97.1)	1 (100.0)	34	1	2
S. lutetiensis	1 (14.3)	4 (71.4)	0 (71.4)	0 (71.4)	0 (71.4)	0 (71.4)	1 (85.7)	1 (100.0)							7	0.015	NC[b]
S. bovis	2 (66.7)					1 (100.0)									3	NC	
S. equinus		2 (100.0)													2	NC	

[a]Clinical isolates selected for further analysis with respective lefamulin MICs are highlighted.
[b]NC, not calculated.

TABLE 2 MICs obtained for lefamulin and comparator agents tested against isolates included in the study[a]

Collection no.	Species	MIC (μg/ml) by agent						
		Erythromycin	Clindamycin	Q-D	Linezolid	Lefamulin	Retapamulin	Chloramphenicol
975498	*S. aureus*	>8	>64	0.5	1	0.5 (0.5)	≤0.06	16
981256	*S. aureus*	0.12	≤0.5	0.5	1	0.5 (0.5)	0.5	4
924825	*S. aureus*	>8	≤0.5	0.5	1	1 (1)	2	8
953474	*S. aureus*	>8	≤0.5	0.5	0.5	1 (1)	1	4
879822	*S. aureus*	0.12	≤0.5	0.5	1	2 (>1)	4	8
913640	*S. aureus*	>8	>64	0.5	1	2 (>1)	2	8
934242	*S. aureus*	0.12	≤0.5	0.5	0.5	2 (1)	2	8
950457	*S. aureus*	0.12	8	1	1	4 (2)	>8	4
916083	*S. aureus*	>8	>64	1	0.25	16 (>1)	8	8
976441	*S. aureus*	>8	>64	4	0.5	32 (16)	>8	64
972481	*S. aureus*	4	4	1	1	>32 (>16)	>8	4
939671	*S. cohnii*	>8	≤0.5	1	1	0.5 (2)	1	4
939504	*S. epidermidis*	>8	≤0.5	≤0.25	16	0.5 (1)	0.25	16
947675	*S. epidermidis*	>8	16	≤0.25	0.5	1 (0.5)	>8	4
951555	*S. epidermidis*	>8	>64	4	0.5	1 (0.5)	1	4
955639	*S. epidermidis*	0.12	≤0.5	0.5	0.5	1 (1)	1	4
956923	*S. epidermidis*	>8	16	≤0.25	0.25	2 (0.5)	>8	2
949426	*S. epidermidis*	≤0.06	1	≤0.25	0.5	2 (2)	>8	4
938399	*S. epidermidis*	>8	≤0.5	≤0.25	0.5	8 (4)	8	2
952506	*S. epidermidis*	>8	1	≤0.25	0.5	8 (4)	8	4
958510	*S. epidermidis*	≤0.06	2	≤0.25	1	8 (2)	>8	4
934123	*S. epidermidis*	0.5	>64	1	128	32 (8)	>8	64
939969	*S. haemolyticus*	>8	>64	4	2	4 (4)	4	32
944662	*S. sciuri*	>8	>64	0.5	1	16 (8)	8	32
941213	*S. sciuri*	0.25	≤0.5	1	1	32 (>16)	>8	4
960742	*S. lutetiensis*	0.03	≤0.5	1	1	0.5 (0.5)	0.5	2
982012	*S. lutetiensis*	≤0.015	1	0.5	1	2 (1)	2	2
971459	*S. agalactiae*	>32	>64	1	1	8 (8)	8	2
935557	*S. agalactiae*	0.03	4	0.5	1	8 (8)	4	2
935554	*S. agalactiae*	0.03	4	0.5	0.5	16 (16)	4	2
965031	*S. gallolyticus*	>32	4	1	2	32 (>16)	>8	4

[a]Values within parentheses are the initial lefamulin MICs obtained during the surveillance studies. Q-D, quinupristin-dalfopristin.

μg/ml) were selected for further evaluation (Tables 1 to 3). All but 1 of the selected streptococci carried *lsa*(E) (lefamulin MIC, 2 to 32 μg/ml) (Table 3). The *S. lutetiensis* isolate with a lefamulin MIC of 0.5 μg/ml carried *lnu*(C) and had a T225C and an A2360G in the 23S rRNA, while alterations within the ribosomal proteins evaluated were not detected (Table 3).

DISCUSSION

This 2-year (2015 to 2016) global surveillance program documents a small number of isolates showing a non-wild-type phenotype for lefamulin. Variants of the *vga* gene (8/11; 72.7%) were observed among most *S. aureus* isolates with lefamulin MICs above the ECOFF value (>0.25 μg/ml), while 2 isolates carried *lsa*(E) or L4 mutations (V118A and E147K). Two *S. aureus* (975498 and 981256) isolates displayed a lefamulin MIC of 0.5 μg/ml, but only the latter carried a *vga*(A) gene. Isolate 975498 only showed alterations in the 23S rRNA that was also observed in other *S. aureus* isolates included in the study, which are likely polymorphisms and not associated with pleuromutilin-resistance phenotypes. Furthermore, these locations are not associated with drug binding (7).

Staphylococci exhibiting elevated MICs to pleuromutilins, lincosamides, and streptogramin A (PLS_A) usually carry the ATP-binding cassette F (ABC-F) proteins, such as those belonging to Vga, Lsa, or Sal families (15–17). In fact, similar to *S. aureus*, *vga* gene variants were also observed among most CoNS (8/14; 57.1%) or in 64.0% (16/25) of all staphylococci selected herein for further investigation. However, studies have demonstrated that alterations in the 23S rRNA and L3 ribosomal protein can also be responsible for decreased susceptibility to pleuromutilins (10–13, 18, 19), but in general, except for some polymorphisms observed in 23S rRNA, the *S. aureus* isolates included here showed 23S rRNA and ribosomal protein sequences equivalent to the respective reference strain.

TABLE 3 Molecular epidemiology and resistance mechanism results for isolates included in this study

Species	Isolate no.	Yr	MLST[a]	Country	Lefamulin MIC (μg/ml)	Resistance determinants[b]		Ribosomal mutations[c]			
						Pleuromutilins	Other	23S rRNA	L3	L4	L22
S. aureus	975498	2016	5	United States	0.5		*erm*(A)	A21G, A1557T	WT	WT	WT
S. aureus	981256	2016	4335	New Zealand	0.5	*vga*(A)		A21G, A1557T, A2234G	WT	WT	WT
S. aureus	924825	2015	88	Australia	1	*vga*(A)	*erm*(C)	A21G, A1557T, A2234G	WT	WT	WT
S. aureus	953474	2016	398	France	1	*vga*(A)	*erm*(T)	A21G, A1557T, A2234G	WT	WT	WT
S. aureus	879822	2015	1	Slovenia	2	*vga*(A)		A21G, A2234G	WT	WT	WT
S. aureus	913640	2015	1148	United States	2	*vga*(A)	*erm*(C)	A21G, A2234G	WT	WT	WT
S. aureus	934242	2016	1148	United States	2	*vga*(A)		A21G, A2234G	WT	WT	WT
S. aureus	950457	2016	97	United States	4	*vga*(A)		A21G, A2234G	WT	WT	WT
S. aureus	916083	2015	5	Korea	16		*erm*(A)	A21G, A1557T, A2234G	WT	V118A, E147K	WT
S. aureus	976441	2016	398	Brazil	32	*lsa*(E)	*erm*(T), *lnu*(B)	A21G, A1557T, A2234G	WT	WT	WT
S. aureus	972481	2016	398	Germany	>32	*vga*(E)	*erm*(A)	A21G, A1526G, A1557T	WT	WT	WT
S. cohnii	939671	2016	N/A	United States	0.5		*msr*(A)	A107G, A124G, T266G, C450T, T623C, A816G, T1261C, T1448A, T1549A	D108E, T190A, N193K, Y208F	N20S, A128T, A133T, V155I	WT
S. epidermidis	939504	2016	2	Italy	0.5		*msr*(A)	G105A, G241T, T669C, T1236C, G2576T	WT	WT	WT
S. epidermidis	947675	2016	57	United States	1	*vga*(A)	*mph*(C), *msr*(A)	G241T, T669C, T1236C	WT	WT	WT
S. epidermidis	951555	2016	87	Czech Republic	1	*vga*(A), *vga*(B)	*erm*(A)	C139T, G241T, T669C, T1236C	WT	WT	WT
S. epidermidis	955639	2016	87	Italy	1	*vga*(A), *vga*(B)	*vat*(B)	C139T, T669C, T1236C, C2809T	WT	WT	WT
S. epidermidis	956923	2016	679	Brazil	2	*vga*(A)	*msr*(A)	T669C, T1236C	V188I	WT	WT
S. epidermidis	949426	2016	255	United States	2	*vga*(A)		T669C, T1236C	WT	WT	WT
S. epidermidis	938399	2016	5	United States	8	*vga*(A)	*mph*(C), *msr*(A)	C139T, T669C, T1236C	WT	WT	WT
S. epidermidis	952506	2016	20	Argentina	8	*vga*(A)	*mph*(C), *msr*(A)	C139T, T669C, T1236C, C1638T	WT	WT	WT
S. epidermidis	958510	2016	487	United States	8	*vga*(A)		G241T, T669C, T1236C, C1638T	WT	WT	WT
S. epidermidis	934123	2016	5	United States	32	*cfr*		T669C, T1236C, C2534T	H146Q, V154L, A157R	G71_R72insG	WT
S. haemolyticus	939969	2016	3	Mexico	4	*cfr*	*mph*(C), *msr*(A), *erm*(C)	C1486T, A2235G, T2882C	WT	WT	A29T
S. sciuri	944662	2016	N/A	Mexico	16	*sal*(A)	*erm*(C)	WT	WT	WT	A112D
S. sciuri	941213	2016	N/A	Australia	32	*sal*(A)		WT	WT	WT	WT
S. lutetiensis	960742	2016	N/A	Belgium	0.5		*lnu*(C)	T225C, A2360G	WT	WT	WT
S. lutetiensis	982012	2016	N/A	Argentina	2	*lsa*(E)	*lnu*(B), *lnu*(C)	WT	WT	WT	WT
S. agalactiae	971459	2016	19	Korea	8	*lsa*(E)	*lnu*(B), erm(B)	WT	WT	R1del, R2K	WT
S. agalactiae	935557	2016	19	Mexico	8	*lsa*(E)	*lnu*(B), erm(B)	WT	WT	R1del, R2K	WT
S. agalactiae	935554	2016	19	Mexico	16	*lsa*(E)	*lnu*(B), erm(B)	WT	WT	R1del, R2K	WT
S. gallolyticus	965031	2016	N/A	Spain	32	*lsa*(E)	*lnu*(B), erm(B)	C696T	WT	WT	WT

[a]MLST, multilocus sequence typing; N/A, not available.
[b]MLS_B (macrolide-lincosamide-streptogramin B) and pleuromutilin resistance genes screened as available at https://faculty.washington.edu/marilynr/ermwebA.pdf.
[c]23S rRNA mutational analysis performed on nucleotide sequences (*Escherichia coli* numbering). Protein sequences analyzed for annotating L3, L4, and L22.

Isolate 916083 displaying a lefamulin MIC of 16 μg/ml was the only *S. aureus* isolate with alterations in L4 (V118A and E147K). This isolate also exhibited elevated MICs for clindamycin, retapamulin, and erythromycin (Table 2). Previous studies linked L4 alterations with decreased susceptibility to tiamulin, chloramphenicol, and oxazolidinones (14, 20, 21). However, these previously reported alterations surrounded position K68, which is relatively close to the PTC and is responsible for stabilizing this region. V118 and E147 at L4 are located far from the PTC, but a hypothesis would be that L4 mutations may perturb the 3-dimensional structure of the 23S rRNA and minimize drug interaction (22). In fact, non-wild-type lefamulin MICs were obtained against 3 *S. aureus* surveillance isolates included in the SENTRY Program for 2010, and further investigations detected only the presence of L4 alterations in these older isolates. These isolates belonged to ST59 (lefamulin MIC, >16 μg/ml), carried A50G and V118A at L4 or ST398 (lefamulin MIC, 16 μg/ml), and had V118A and V142I at L4 (unpublished data). These data suggest that V118A, common to these *S. aureus* isolates from 2010, 2015, and 2016, may be associated with a decreased susceptibility to this agent. However, additional studies are needed to truly link this L4 alteration with the MICs presented here.

Two *S. sciuri* isolates showed lefamulin MICs of 16 to 32 μg/ml and did not carry any acquired resistance genes associated with the pleuromutilin phenotype. However, the *sal*(A) gene was detected in both isolates, and this gene was previously determined to be ubiquitous in this species and to cause decreased susceptibility to pleuromutilins and other agents (15, 23, 24). In addition, this gene has been detected in several staphylococcal species other than *S. sciuri* from animal and human origins, indicating that it has been mobilized to other bacterial species (15).

Both CoNS (939504 and 939671) isolates with a lefamulin MIC of 0.5 μg/ml had multiple alterations in the 23S rRNA. A G2576T was noted in isolate 939504, which is a well-known oxazolidinone resistance mechanism (14, 25) and known to affect tiamulin and valnemulin binding (11, 26). The binding effect for lefamulin appears to be less pronounced, likely because lefamulin appears to have more hydrogen bonds formed at the binding site than tiamulin and valnemulin. It also does not interact directly with G2576, although an alteration at this position causes a shift at the backbone of nucleotides from positions 2504 to 2507 (11), and these nucleotides interact with lefamulin (7). Importantly, isolate 939504 displayed a linezolid MIC of 16 μg/ml (Table 2), indicating the presence of G2576T in several 23S rRNA alleles (14); therefore, the lower lefamulin MIC was likely caused by a minimal effect of G2576T on drug binding rather than a low number of mutated ribosomes. As additional evidence, several staphylococci isolates included in past years of the SENTRY Program were characterized because of elevated linezolid MICs (≥8 μg/ml). Those isolates showing only G2576T had lefamulin MICs of 0.12 to 0.5 μg/ml (unpublished data), which are at the right side of the modal MIC (0.06 μg/ml) for *S. aureus* (Table 1). All 23S rRNA alterations observed in isolate 939671 are located outside the lefamulin binding site (13), and the L3 and L4 alterations detected have not been previously associated with resistance (19).

One *S. epidermidis* (lefamulin MIC, 32 μg/ml) and 1 *S. haemolyticus* (lefamulin MIC, 4 μg/ml) isolate carried *cfr*. This transferable gene confers a resistance phenotype to several classes of drugs (9), and its dissemination could jeopardize the clinical utility of several agents used in humans and animals. These study results corroborate those from large surveillance investigations that documented a low prevalence of *cfr* among Gram-positive isolates (25, 27, 28). Several studies reported sporadic outbreaks of *cfr*-carrying staphylococci; however, it was documented that the dissemination of such isolates are usually controlled by a combination of antibiotic stewardship and infection control measures (29–32).

All but 1 of the 6 streptococcal isolates selected for this study carried *lsa*(E). This gene has been reported among several Gram-positive isolates recovered from human and animal specimens (15, 24, 33). *lsa*(E) is usually part of a gene island that includes several resistance genes, including *lnu*(B) upstream (24, 34), which confers resistance to lincosamides. Among selected streptococcal isolates, pleuromutilin resistance mecha-

nisms were not detected in *S. lutetiensis* 960742, except for 2 alterations in 23S rRNA (T225C and A2360G) located outside the lefamulin binding site (13). Isolate 960742 displayed a lefamulin MIC of 0.5 μg/ml, which is 32-fold higher than the modal MIC (0.015 μg/ml) shown for this species (Tables 1, 2).

In summary, this study showed a low prevalence of isolates exhibiting a non-wild-type MIC for lefamulin among Gram-positive isolates included in a 2-year global surveillance program. The non-wild-type phenotypes observed here could be generally explained by the presence of *vga* in staphylococci and *lsa*(E) in streptococci, which are more often detected among isolates collected from animals (15, 33–37). The association of acquired genes detected here with isolates from animal origins is further evidenced by the presence of 3 *S. aureus* isolates belonging to ST398, a lineage commonly responsible for infections in animals (16, 36–39). This study benchmarks the lefamulin activity against a global contemporary collection of Gram-positive surveillance isolates, as well as the rare instance of resistance genes associated with decreased susceptibility before clinical approval and use of this unique agent of the pleuromutilin class. Although the prevalence of surveillance isolates exhibiting non-wild-type MICs for lefamulin are rare, continued surveillance to monitor transferable genes and changes in MIC over time will provide valuable information for this new class of antibacterial agents for humans.

MATERIALS AND METHODS

Clinical isolates. A total of 3,195 staphylococci and 4,489 streptococci isolates were included as part of the lefamulin surveillance program from 2015 to 2016. Based on the MIC distributions shown in Table 1, ECOFF values ($\leq$0.25 μg/ml for *S. aureus* and CoNS) were calculated to define the lefamulin wild-type population of *S. aureus* and CoNS that included 99.9% of isolates within each group (40). Thus, staphylococcal isolates exhibiting lefamulin MICs of $>$0.25 μg/ml were selected for further molecular characterization (Table 1). Streptococci were selected based on species, and those isolates displaying elevated lefamulin MICs within a given species were selected for further molecular characterization (Table 1). Bacterial isolate identification was confirmed by matrix-assisted laser desorption ionization–time of flight mass spectrometry (Bruker Daltonics, Bremen, Germany) and genome sequencing.

Antimicrobial susceptibility testing. Isolates were tested for susceptibility by broth microdilution methods, according to the recommendations of the Clinical and Laboratory Standards Institute (CLSI) (41). Frozen-form broth microdilution 96-well plates were manufactured by JMI Laboratories and contained cation-adjusted Mueller-Hinton broth (2.5% to 5% lysed horse blood added for testing streptococci). Isolates that met the inclusion criteria were retested for susceptibility in frozen-form panels containing extended ranges for lefamulin among other agents (Table 2). Bacterial inoculum density was monitored by colony counts to ensure an adequate number of cells for each testing event. MICs were validated by concurrently testing CLSI-recommended quality-control strains (42).

Characterization of resistance mechanisms by next-generation genome sequencing and analysis. Selected isolates had total genomic DNA extracted with the fully automated Thermo Scientific KingFisher Flex magnetic particle processor (Cleveland, OH, USA), which was used as input material for library construction. DNA libraries were prepared using the Nextera library construction protocol (Illumina, San Diego, CA, USA) following the manufacturer's instructions and were sequenced on a MiSeq sequencer (JMI Laboratories, North Liberty, IA, USA). FASTQ format sequencing files for each sample set were assembled independently using the *de novo* assembler SPAdes 3.9.0 (43), and an in-house-designed software program was applied to the assembled sequences to align against known macrolide-lincosamide-streptogramin B (MLS_B) and pleuromutilin resistance genes, including *tva*(A) (44–46).

Sequences of 23S rRNA (PTC), *rplC* (L3), *rplD* (L4), and *rplV* (L22) were extracted from the assembled sequences and evaluated against corresponding sequences of susceptible wild-type reference strains. The analysis of 23S rRNA was performed based on nucleotide sequences (*Escherichia coli* numbering), while those from rRNA proteins were based on amino acid sequences. All intrinsic 23S rRNA target genes or ribosomal protein amino acid sequences were considered wild type if 100% identity was observed with the respective reference sequences. Nucleotide and amino acid differences were annotated when an identity of $<$100% was observed. Reference sequences were extracted from the following strains: *S. aureus* (NCTC 8325), *S. epidermidis* (ATCC 12228), *S. cohnii* (ATCC 29974), *S. haemolyticus* (JCSC1435), *S. sciuri* (ATCC 29062), *S. lutetiensis* (NCTC 13774), *S. agalactiae* (NEM316), and *S. gallolyticus* (ATCC 43143).

Multilocus sequence typing. Multilocus sequence typing (MLST) was performed by extracting previously defined sets of 7 housekeeping gene fragments (approximately 500 bp) from each assembled sequence. Each fragment was compared with known allelic variants for each locus (housekeeping gene) on the MLST website (PubMLST, https://pubmlst.org). An allele sharing 100% genetic identity with a known variant received a numeric designation, and a 7-number sequence (1 for each housekeeping gene) formed an allelic profile, defined as STs.

Data availability. This is an original work and the data set repository and published article in which the data set and/or code was originally described and have not been published previously. Upon request, and subject to certain criteria, conditions and exceptions, JMI Laboratories and Nabriva Therapeutics will

provide access to the code and databases utilized here. This information may be requested 24 months after study completion and will be made available to researchers whose proposals meet the research criteria and other conditions and for which an exception does not apply, via a secure portal. To gain access, requestors must enter into an information access agreement with JMI Laboratories and/or Nabriva Therapeutics.

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